DISCUSSION

Drug–resistant TB emerges as a result of TB treatment mismanagement, and is passed from person to person in the same way as drug sensitive TB. MDR- TB is caused by bacteria that are resistant to the most effective anti-TB drugs (ionized and rifampicin). MDR-TB results from either primary infection or may develop in the course of patient’s treatment. The WHO’s Global tuberculosis report 2012, says that of the 73,000 estimated MDR TB patients living in India, only 1,660 cases were notified and 1,136 cases were put on treatment.

The pattern of drug resistance varies from place to place and at different period of time. It is important to know the drug resistance pattern in that area for a better treatment. MDR-TB was associated with high death rates of 50–80%, spanning a relatively short time from diagnosis to death \[162\]. Drug resistance is a result of random genetic mutations in particular genes. Delay in the recognition of drug resistance results in a delay in initiation of treatment or effective chemotherapy, which is the major factor that contributes to MDR-TB outbreaks. Drug resistance now can be assessed rapidly within 1 day by a molecular assay. In this study we reviewed our drug sensitivity data of mycobacterium tuberculosis. In our study we found 23.33\% (35/150) MDR –TB from pulmonary tuberculosis patients. Out of which 80\% strains were resistant to streptomycin (STR), isoniazid (INH), rifampicin (RIF), and ethambutol (EMB). Menon et al, in 2014, found 17.53\% strains were resistant to all four drugs in Mumbai \[163\].
Mutations in the katG and the inhA genes are associated with approximately 70% and 80% of INH-resistant MTB isolates \[164\]. Although isoniazid resistance in \textit{M. tuberculosis} is more complex due to the implication of the number of genes, up to 95% of isoniazid resistance may be due to mutations in katG \[165\]. Twenty (57.14\%) of the MDR isolates showed a mutation in the katG codon 315 and fifteen (42.86\%) of the MDR isolates showed a mutation in inhA gene. The prevalence of mutations in the katG and inhA genes seems to vary widely in different geographic locations. Ninety-seven percent of katG mutations and 24\% of inhA mutations were found in the INH-resistant isolates from KwaZulu-Natal \[166\], whereas Van Rie \textit{et al}, 2001 reported 72\% of katG mutations and 2\% mutations in the inhA gene of INH-resistant isolates in the Western Cape Province of South Africa \[167\]. Extensive studies from other countries have confirmed this variability in the contribution of different mutations to INH resistance \[168, 169\]. The genotype MTBDR \textit{plus} assay can detect the low level of isoniazid resistance associated with inhA gene, which was not possible with the previous version of the assay (genotype MTBDR assay), which could only detect the mutations in the katG gene.

Rif-resistance is almost exclusively associated with mutation of the rpoB gene, which encodes the β-subunit of RNA polymerase \[170, 171\]. More than 95\% of rpoB mutations in Rif-resistant clinical isolates have been found within RRDR. Over 70 distinct rpoB mutations and four frequent mutation (codon 531, 526, 516, 513) has been reported for RIF–resistant \textit{M. tuberculosis} isolates worldwide \[172, 173\]. Rif- resistance is often regarded as an excellent surrogate marker for MDR tuberculosis \[23, 174\]. In our study all 35 (23.33\%) isolates show resistance to both rifampicin and isoniazid and having a mutation in 81-bp RRDR of rpoB gene. Previously characterized mutations were defined as those associated with drug resistance as listed in the Tuberculosis drug resistance mutation database \[175\]. Mani \textit{et al}, in 2001, found 88\%
(44/50) rifampicin resistant isolates from various parts of India \[176\]. Khanna et al, in 2010, found 54\% (104/194) MDR-TB in Delhi & neighbouring regions in India \[177\]. Multidrug resistant tuberculosis (MDR-TB) cases have shown a worrying increase in the eight southern districts of Panjab, India in the past one year. The disease has also shown almost 100\% rise in eight southern districts – Patiala, Mohali, Fatehgarh sahib, Ludhiana, Barnala, Sangrur, Mansa & Roopnagar (India) \[178\].

In previous studies most of the rpoB mutations found in \textit{M. tuberculosis} strains were single amino acid substitution (single point), at either position Ser-531 (Leu/Gln) or His-526 (Asp/tyr); although for a few strains, double mutation \[179, 180\], and even triple mutation existed \[179\]. In contrast to this, in our study we found 34\% (12/35) of strains had mutation at more than one position in the rpoB gene and mutation frequencies at different position were also different from the earlier studies. In our study we observed that the \textit{M. tuberculosis} isolates with the Rif\textsuperscript{r} phenotype contain missense mutation that show amino acid substitution at Ser-531(40\%), His-526 (23\%) and Asp-516 (15\%) residues. This finding is almost similar to result reported by Ramaswamy and Musser; USA, 1998 \[18\], who showed frequencies of 41 and 36\% for various mutation occurring at codon 531 and 526 respectively, in 478 isolates obtained from various part of the world. Matsiota-Bernard et al, 1998 \[181\] and Pozzi et al, 1999 \[179\] found high frequencies of mutation at codon 531, 53\% and 59\% respectively. Although Qian et al, 2002 \[182\], reported a low frequency of the mutation at codon 526(4\%) in china isolates, similarly a low frequency of the mutation (7\%) at this codon also reported in Panjab, Pakistan \[183\]. we found high frequency of this mutation (23\%). Similarly high frequencies of this mutation at codon 526 have found in isolates from Delhi (26\%) \[184\], Hyderabad (17\%) \[185\], North India (20\%) \[186\], Japan (33\%) \[182\], Greece (19\%) \[181\], Italy (30\%) \[179\], Korea (38\%) \[180\]. Distribution of
mutations in the RRDR of the rpoB gene in MDR *M. tuberculosis* from different geographic region of India is also different \[187\].

In our study, 23 (66%) of 35 strains had a mutation at only one position, and 34% (12/35) of strain had mutation at more than one position in the rpoB gene and mutation. Study in Hyderabad showed 2 strains out of 30 samples with multiple mutations \[185\] and 13.33% isolates have multiple mutations in RRDR region out of 30 samples reported in North India \[186\] and in Delhi on 50 samples do not found any strain with multiple mutations \[184\].

In this study molecular analysis of 81-bp RRDR of rpoB gene of *M. tuberculosis* clinical isolates were carried out & mutations were identified. New mutations reported in this study include mutation from AGC (Ser)-TGC (Cys) at codon 512, TTC (Phe)-TTT (Phe) at codon 514, CAG (Gln)-CAA (Gln) at codon 517, AAC (Asn)- ACC (Thr) at codon 519, TTG (Leu)-ATG (met) at codon 524. Despite the large number of mutations already reported in other studies, the evidence of new mutations in this study indicates that mutations continue to arise, probably due to the ability of *M. tuberculosis* to adapt to drug exposure. For the control of MDR–TB, elimination of uncompleted treatment is critical. Since uncompleted treatment tends to facilitate the rate of evolution of increased levels of drug resistance within MDR-TB clones \[188, 189\], which facilitates increase in prevalence of such clones in *M. tuberculosis* populations, treatment become more difficult, which makes non-compliance more likely, and therefore uncompleted treatments more likely, which completes a vicious self-accelerating cycle. This possibility of uncompleted treatments resulting in escalating rise in prevalence and strength of MDR-TB means that every case of uncompleted treatment counts, and should be avoided if man is to stem
the MDR-TB tide. Hence, early detection of the MDR-TB may well be critical to durable control of tuberculosis.
500 Sputum samples were collected from suspected pulmonary tuberculosis patients at the TB-Chest department of Subharti Medical College, Meerut (U.P. INDIA).

The samples were processed by standard method; Firstly the sputum was subjected to routine Ziehl-Neelsen staining & the rest of the sputum was digested and decontaminated by N-Acetyl-L-Cysteine-2%NaOH method (NALC-2%NaOH) and concentrated by the centrifugation at 3000g for 20 min, from the pellet two Lowenstein-Jensen medium slants were inoculated and incubated at 37˚C for 6-8 weeks. The inoculated LJ media were examined every second day during the first week and then weekly for up to 8 weeks for the presence of growth.

PCR were performed by commercial kit method from Bangalore Genei, Bangalore (India). This test is based on the principle of single–tube nested PCR method, which is very sensitive diagnostic tool for the identification of *M. tuberculosis*. This assay is two–step sequential assay. In the first step, the IS region of MTB complex DNA sequence, a 220 bp is amplified by specific external primers. In the second step, the nested primers are added to further amplify a 123bp amplification product. In sputum sample one aliquot of the sediment obtained after NALC-2%NaOH decontamination was kept frozen at -20˚C was used as a source of DNA.

A total of 150 clinical isolates of M. tuberculosis were obtained from culture and PCR positive pulmonary TB patients.
All the 150 isolates were tested with the BACTEC MGIT 960 SIRE susceptibility test, followed by GenoType MTBDR assay (Hain Life Science GmbH, Nehren, Germany) and DNA sequencing.

As a result of BACTEC MGIT 960 SIRE susceptibility test and GenoType MTBDR assay, (Hain Life Science GmbH, Nehren, Germany) 115 isolates were sensitive to streptomycin (STR), isoniazid (INH), rifampicin (RIF), and ethambutol (EMB) and remaining 35 isolates were found as MDR.

To detect the resistance to rifampicin, mutations in 81-bp rifampicin resistance determining region (RRDR) of rpoB gene in clinically isolated *M. tuberculosis* strains were analyzed by DNA sequencing.

As a result of DNA sequencing of 35 rifampicin resistant strains, rpoB positive PCR products showed that all 35 isolates displayed mutation in the rpoB gene.

Our analysis detected a total 24 mutated positions distributed among 10 codons within RRDR of the rpoB gene.

In conclusion, the rifampicin resistance (RIF\(^r\)) appears to be an effective marker of MDR-TB, and confirmation with PCR-DNA sequencing can detect the presence of RIF\(^r\) *M. tuberculosis* within 2 days, and can clearly differentiate them from RIF\(^s\) strains, the technique described in our study may prove to be useful for identifying MDR-TB.

Our study demonstrate the feasibility and ease of using automated DNA sequencing to rapidly and unambiguously characterize rpoB mutation associated with rifampicin resistance.
Main advantage of this technique is that to allow the identification of all mutations in the target sequence, the analysis of the 81-bp RRDR of rpoB gene of 35 *M. tuberculosis* clinical isolates was performed and mutations were recognized.

Five new mutations were recognized.

Finding of our study highlighted the emerging change in the trend of mutation profile of rpo B gene in rifampicin resistant mycobacterium tuberculosis. Though detection of mutation by DNA sequencing is gold standard, it cannot be used as screening method due to cost and technical complexity of method.

Despite the large number of mutations already reported in other studies, the evidence of new mutations in this study indicates that mutations continue to arise, probably due to the ability of *M. tuberculosis* to adapt to drug exposure.

Knowledge of mutation profile from different geographic area has implication in diagnosis of MDR-TB by developing molecular screening method.

Changing mutation pattern may alter sensitivity and specificity of screening methods which are based on detection of mutation.

However large scale studies with adequate sample size from different geographic areas needed before come to conclusion of changing pattern of mutation profile.

This DNA sequencing can serve two fold functions. First it can provide an accurate and rapid detection of RIF resistant *M. tuberculosis* to be clinically useful and secondly it can provide useful data in developing a screening protocol for detection of MDR-TB in future.